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T-box and homeobox genes from the ctenophore *Pleurobrachia pileus*: Comparison of *Brachyury*, *Tbx2/3* and *Tlx* in basal metazoans and bilaterians

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Abstract **Most animals are classified as Bilateria and only four phyla are still extant as outgroups, namely Porifera, Placozoa, Cnidaria and Ctenophora. These non-bilaterians were not considered to have a mesoderm and hence mesoderm-specific genes. However, the T-box gene *Brachyury* could be isolated from sponges, placozoans and cnidarians. Here, we describe the first *Brachyury* and a *Tbx2/3* homologue from a ctenophore. In addition, analysing T-box and homeobox genes under comparable conditions in all four basal phyla lead to the discovery of novel T-box genes in sponges and cnidarians and a *Tlx* homeobox gene in the ctenophore *Pleurobrachia pileus* . The conservation of the T-box and the homeobox genes suggest that distinct subfamilies with different roles in bilaterians were already split in non-bilaterians.**

Keywords: Bilateria; Ctenophora; T-box; Homeobox; Mesoderm; Evolution

1. Introduction

Zoology textbooks classify multicellular animals often as diploblasts and triploblasts or Radiata and Bilateria. Triploblasts or Bilateria comprise all higher animals from flatworms to humans classified in over 30 phyla. At the base of the evolutionary tree, only four phyla are left, namely Porifera, Placozoa, Cnidaria and Ctenophora. These four phyla are clearly basal to bilaterians, but they are not all diploblastic nor all radial and until further clarification we call them non-bilaterians. The classical division of the animal kingdom has been recently reinvestigated by molecular genetic approaches, which make the division between bilaterians and non-bilaterians appear less clear [1]. For instance, investigating *Hox* and *dpp* gene expressions in the sea anemone *Nematostella vectensis*, it has been shown that the bilateral symmetry arose before the split between cnidarians and bilaterians [2]. At the same time, the division between diploblasts and triploblasts is fading, since genes associated with mesoderm in bilaterians are present and expressed in a similar pattern in cnidarians [3–7]. Most studies consider only one of the four non-bilaterian phyla, the cnidarians; but to study the origin of metazoans it has been proposed that all four basal animal phyla should be considered [8].

One class of genes present in all animals tested so far and absent in all complete non-animal genomes is the T-box family. Recent studies have improved such datasets by isolating T-box genes first from cnidarians [5,9,10], but also from sponges [11,12] and even from placozoans [13]. In order to include the ctenophores in the investigation on T-box genes, we report the isolation of *Brachyury* and *Tbx2/3* homologues from *Pleurobrachia pileus* (sea gooseberry). At the same level of detection, we isolated a novel family member from the sponge *Axinella verrucosa* and a *Tbx4/5* homologue in addition to *Brachyury* [5] from the cnidarian *Podocoryne carnea*.

Even better known than T-box genes are homeobox genes. Although some subfamilies can be found in fungi and plants, the *Hox* cluster was thought to be important for animal evolution. Although a true *Hox* cluster could not be identified in non-bilaterians, many different homeobox genes are known [14]. With material from all four basal phyla we could detect a Hox/ParaHox-type *Gsx* homologue in *Trichoplax* or *Podocoryne* but not in sponges or ctenophores. In addition, in *Trichoplax* a *Not* gene was detected [15] and a single homeobox gene in the ctenophore *Pleurobrachia*, namely a *Tlx* homologue. Although more data are required to enable detailed comparisons with bilaterians, our data suggest that the genome of the ancestral metazoan contained already members of several distinct subfamilies of the T-box as well as the homeobox gene families.

2. Materials and methods

2.1. Animals

P. pileus (Ctenophora, Cydippida) were caught at the Marine Biology Station of Roscoff (France). Adult animals were kept in aquaria for about 10 days, long enough to produce larval stages. These were selected individually and washed with Millipore filtered sea water before nucleic acid extraction. Also a body fragment of *A. verrucosa* (Porifera, Demospongiae) was brought to our laboratory from Roscoff. Cells from *Axinella* were isolated by cutting the fragment in little pieces, then these pieces were washed with Millipore filtered sea water and successively cells were scratched out with forceps. The cells were separated from the rest by a few seconds of centrifugation at 4 °C before nucleic acid extraction. *Trichoplax adhaerens* (Placozoa) and *Podocoryne carnea* (Cnidaria, Hydrozoa) were cultured and processed as described previously [13,4].

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2.2. Molecular cloning and sequence comparison

Genomic DNA and total RNA were isolated with TriReagent (Molecular Research Center) according to the manufacturer's recommendations. First strand cDNA was synthesized with the anchored oligo(dT) primer XT20V (5′-GGC AGG TCC TCG TTG ACT CGA GAC $GT_{(20)}$(AGC)-3′) by using the SMART RACE cDNA Amplification Kit (Clontech). By homology PCR, 3′ and 5′ RACE the full length coding sequence of the following novel genes were isolated and submitted to the DNA databases with the indicated accession numbers: *Tbx1/15/20* (2607 bp; AJ581005) from *Axinella*, *Brachyury* (1731 bp; AJ581007), *Tbx2/3* (2523 bp; AJ581010) and *Tlx* (1355 bp; AJ581009) from *Pleurobrachia* and *Tbx4/5* (1646 bp; AJ581006) from *Podocoryne*.

A 272-bp *Axinella Tbx1/15/20* fragment was amplified with the set of degenerated primers TF1 and TR1 [4], followed by TF2 and TR1 [13]. For PCR standard conditions were used, except that the annealing temperature was 37 °C for the first 20 cycles and in the second PCR the annealing temperature was 37 °C for 10 cycles and 45 °C for 35 cycles. By the same methodology, a 113 bp *Pleurobrachia Brachyury* fragment was amplified with the primers TF1 and TR1, followed by TF2 and TR2. A 185-bp *Pleurobrachia Tbx2/3* fragment was amplified with the primers TF3 (AAT TCA ATG CA(CT) AA(AG) TA(CT) (GC)A(AG) CC) and TR3 (CTG AAT CC(CT) TT(AGCT) GC(AG) AA(AGCT) GG(AG) TT) and the annealing temperatures of 37 °C for 10 cycles and 45 °C for 35 cycles. Under the same conditions, two T-box gene fragments of 664 and 484 bp were isolated from *Podocoryne* genomic DNA. Both fragments contain an intron of 473 and 297 bp, respectively. The first fragment was represented as well in *Podocoryne* cDNA and turned out to be a *Tbx4/5* homologue, the second could not be detected in cDNA (data not shown). With the set of degenerated primers HoxE and HoxF [16] a 83-bp *Tlx* fragment was amplified from *Pleurobrachia* with annealing temperatures of 37 °C for 10 cycles and 50 °C for 40 cycles. PCR products of the expected size were gel purified with a Qiaquick column (Qiagen), subcloned in the pCRII-TOPO vector (TOPO TA cloning Dual Promoter kit, Invitrogen) and sequenced on an ABI PRISM 310 genetic analyser (Applied Biosystems). Based on the sequences gene-specific primers were designed to carry out the 5′ and 3′ RACE on SMART cDNA. Sequence analysis was performed as described previously [13].

3. Results

3.1. Conserved T-box genes from non-bilaterians

Two distinct T-box family members were isolated from the ctenophore *P. pileus*. One is clearly a member of the Brachyury subfamily and the other a member of the Tbx2/3/4/5 subfamily (Fig. 1). Within the T-box domain *Pleurobrachia Brachyury* is 70–80% identical to Brachyury subfamily members and less than 55% identical to other subfamilies while little similarity can be detected outside of the T-box.*Pleurobrachia Tbx2/3* is 60–70% identical to Tbx2/3/4/5 subfamily members in the T-box domain, and less related to other subfamilies.

One T-box family member was isolated from the sponge *Axinella verrucosa*. Although this gene does not clearly belong to any of the known subfamilies from bilaterians (Fig. 1), the T-box domain is 55–65% identical to Tbx2/3/4/5 and Tbx6/16 subfamilies and less than 48% identical to Brachyury and Eomes subfamilies. No sequence similarity is present outside the T-box domain.

Two distinct T-box family members were isolated from the cnidarian *Podocoryne carnea* by homology PCR on genomic DNA in addition to the already cloned *Brachyury* homologue [5]. One of the gene fragments could not be extended on cDNA and the incomplete sequence gives a weak indication that it could belong to the Tbx1 subfamily (data not shown). The other gene, *Podocoryne Tbx4/5*, is clearly a member of the Tbx2/3/4/5 subfamily (Fig. 1). In the T-box domain it is 65–75% identical to the genes of this subfamily, while it is less than 55% identical with other members of the T-box family.

Phylogenetic analyses of T-box genes were performed by the neighbour-joining methods (N-J) with Clustal X [17] and by the maximum likelihood method (M-L) with TREE-PUZZLE [18] (Fig. 1). T-box genes were selected from representative phyla and the trees were calculated using only the T-box domain of each protein. The phylogenetic analyses confirm the data obtained from the sequence comparisons.

3.2. Identification of the Pleurobrachia homeobox gene Tlx

Repeated homology PCR on *Pleurobrachia* cDNA and genomic DNA revealed just one homeobox gene, namely a *Tlx* homologue. *Pleurobrachia Tlx* is the first full length homeobox gene isolated in ctenophores and has 50–60% of sequence identity with members of the Tlx family in the homeodomain, while there is less than 50% identity with *Lbx* and other homeobox genes. *Tlx* genes encode highly related homeodomain sequences and share the eh1 repression domain at the amino-terminal region (Fig. 2A). Phylogenetic analysis based on the homeodomain confirms that *Pleurobrachia Tlx* belongs to a well-defined homeobox gene subfamily. It is more similar to other *Tlx* genes than to *Lbx* or *Nk2* genes (Fig. 2B), which appear as most related subfamilies in a larger phylogenetic analysis of non-bilaterian homeobox sequences [14].

4. Discussion

Based on their simplicity, it has been assumed that ctenophores represent an ancient metazoan taxon and due to their similarity with jellyfish they were believed to form a phylum together with cnidarians, the so-called coelenterates. It is now accepted that ctenophores are a phylum of their own. Their developmental stages are unique and have been well-characterized in the species *Mnemiopsis leidyi* [19]. Ctenophore 18S rRNA genes show a low level of genetic variability compared to other phyla [20], suggesting that extant ctenophores are all derived from a relatively recent common ancestor. The only full length protein-coding gene analysed so far in ctenophores was the *forkhead* homologue *ctenoBF1*, which is expressed in the mouth and feeding apparatus of *Mnemiopsis* [21].

Besides these few data, ctenophores have not been much investigated at the molecular level in comparison with the other basal phyla. Sponges, cnidarians and recently even placozoans get more attention; probably simply because they are easier to keep under laboratory conditions. However, ctenophores are complex mobile animals and represent an important alternative outgroup to bilaterian evolution and should be included in comparative analyses. This should be possible on a genome scale [22] or as a first approach with an EST analyses which can be performed even on tentacles of a jellyfish [23]. We show here that a molecular analysis is possible with highly conserved developmental genes such as T-box or homeobox genes.

Members of the T-box gene family have been found in all the animal phyla so far investigated [24], but no T-box gene can be recognized in genomes of fungi, plants or parasitic protists. The main feature of the proteins encoded by T-box genes is a conserved region of about 180 amino acids, called the T-box domain. Within the T-box gene family there is still some confusion about the classification in five to eight subfamilies and the nomenclature in different species [24,25], but at least

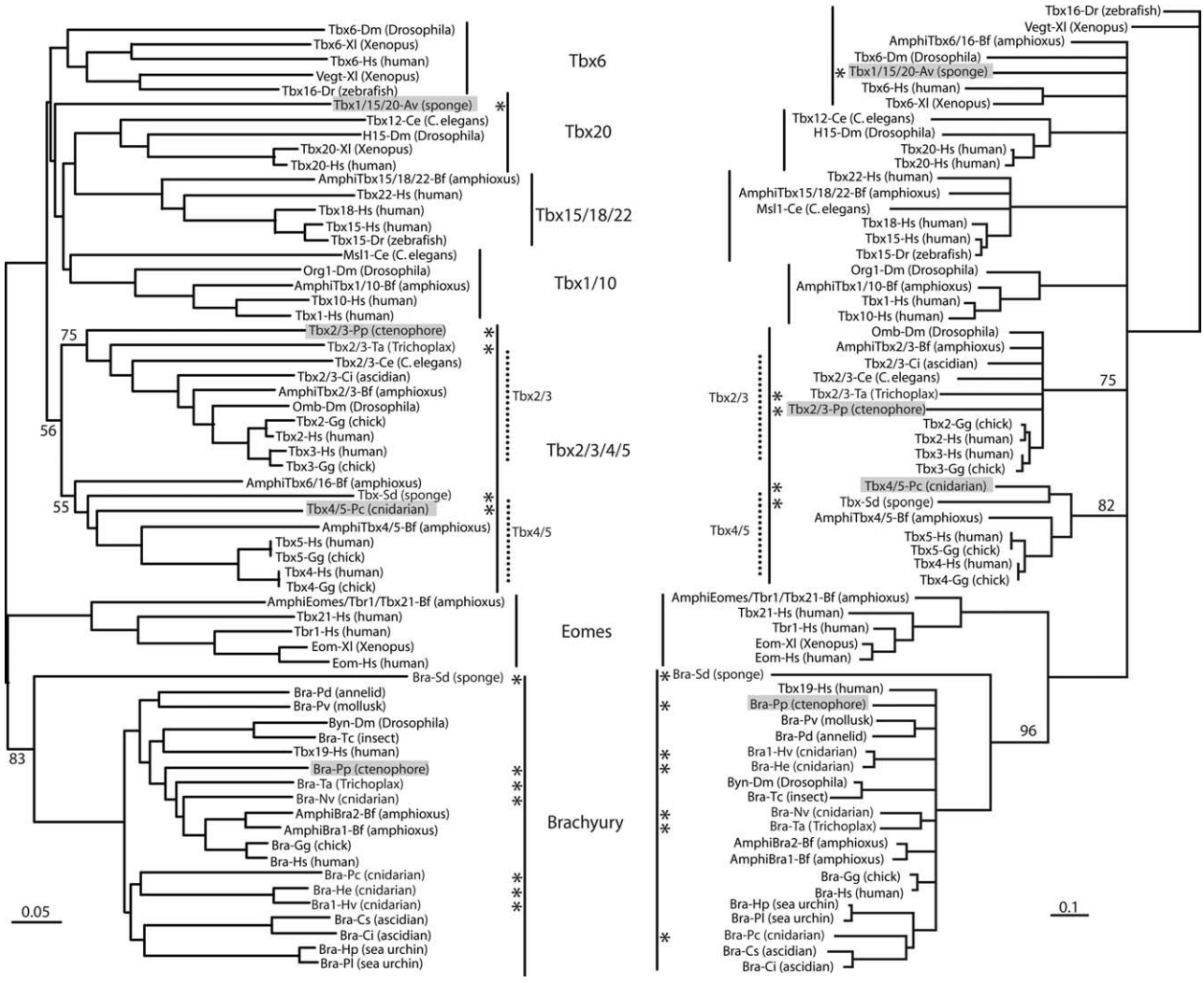

Fig. 1. T-box genes of ctenophores and other non-bilaterians compared to bilaterian T-box subfamilies. The T-box gene family is divided into seven subfamilies; the Tbx2/3/4/5 subfamily can be split further in the Tbx2/3 and Tbx4/5 groups. At least three of these subfamilies contain T-box genes isolated from non-bilaterians (labelled with asterisks; genes described in this study highlighted by shading). The tree on the left was obtained with the N-J, the tree on the right with the M-L method. Numbers on the branches indicate the percentage of 1000 bootstrap replicates that support the topology shown. Bars represent the number of substitutions per site. Abbreviations: Dm, *Drosophila melanogaster*; Xl, *Xenopus laevis*; Hs, *Homo sapiens*; Dr, *Danio rerio*; Av, *Axinella verrucosa*; Ce, *Caenorhabditis elegans*; Bf, *Branchiostoma floridae*; Pp, *Pleurobrachia pileus*; Ta, *Trichoplax adhaerens*; Ci, *Ciona intestinalis*; Sd, *Suberites domuncula*; Pc, *Podocoryne carnea*; Pd, *Platynereis dumerilii*; Pv, *Patella vulgata*; Tc, *Tribolium castaneum*; Nv, *Nematostella vectenis*; He, *Hydractinia echinata*; Hv, *Hydra vulgaris*; Cs, *Ciona savignyi*; Hp, *Hemicentrotus pulcherrimus*; Pl, *Paracentrotus lividus*.

comparisons of amphioxus and vertebrates suggest a quite conservative evolution of this gene family within chordates [25]. Our phylogenetic analyses suggest the presence of seven subfamilies of T-box genes and this division is consistent in both N-J and M-L analyses (Fig. 1). These subfamilies form two larger groups of genes; in one group are the *Brachyury* and *Eomes* gene subfamilies, in the other group are the remaining Tbx-subfamilies and both groups are already represented in non-bilaterians.

Sequence comparisons and phylogenetic analyses show clearly that the two T-box genes isolated from *Pleurobrachia* belong to the Brachyury and Tbx2/3/4/5 subfamilies, respectively. The Tbx2/3 subtree calculated by the N-J method indicate that *Pleurobrachia Tbx2/3* is basal to the other members of this group of genes and placed near to the *Trichoplax* homologue, suggesting an early branching of ctenophores in the evolutionary history. On the contrary, *Pleurobrachia Brachyury* is not basal in the Brachyury subtree; but it was previously shown that phylogenetic analysis of *Brachyury* does not conform to any reasonable expectation [13]. It is unlikely that this subtree would be informative in relation to the evolutionary history of the phyla represented.

Pleurobrachia Tbx2/3 and *Podocoryne Tbx4/5* appear to be in two different subgroups of the Tbx2/3/4/5 subfamily. Members of the Tbx2/3 subgroup were found before in *Trichoplax* [13] and several invertebrates. Tbx4/5 members were thought to be the result of a tandem duplication at the origin of chordate evolution [26] followed by genome duplication in vertebrates [27]. Besides amphioxus, no invertebrate Tbx4/5 members were known until the discovery of a sponge [11] and the *Podocoryne* homologue. This could suggest now that the tandem duplication had occurred before the separation of modern phyla and some phyla kept only one, the other or both duplicates.

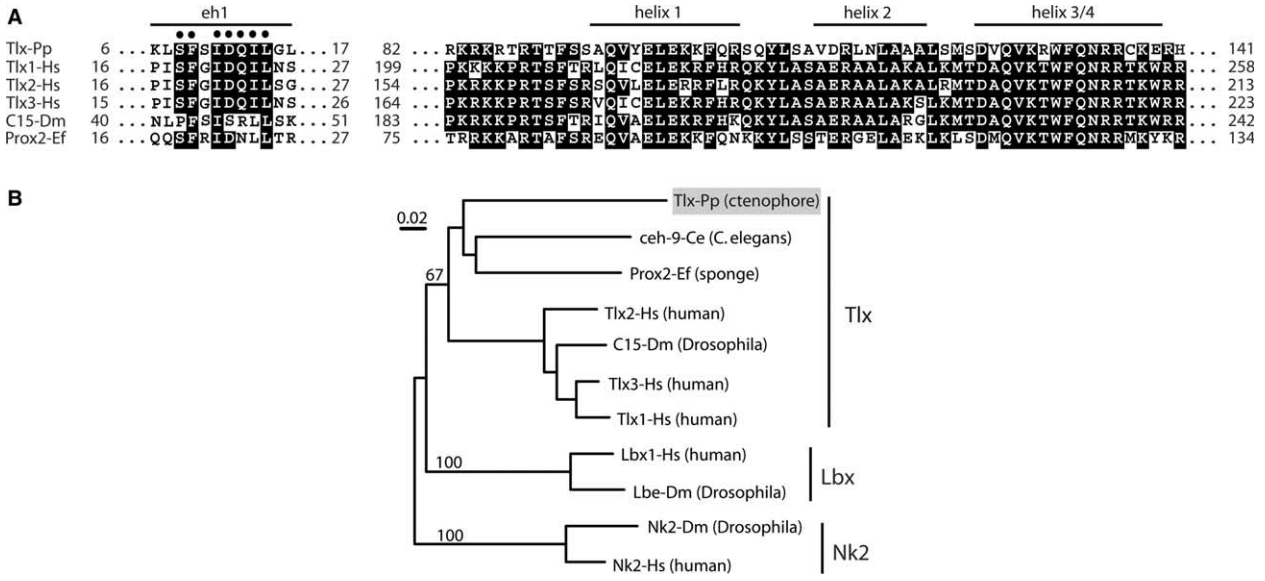

Fig. 2. Sequence comparison of the homeobox gene *Tlx*. (A) The sequence alignment shows the two regions most conserved in *Tlx* genes, the eh1 motif and the homeodomain. Conserved residues in the *Pleurobrachia* eh1 motif are highlighted with a dot. (B) A phylogenetic tree based on the N-J method shows clearly that *Pleurobrachia Tlx* belongs to the Tlx subfamily with *Lbx* and *Nk2* genes as outgroups. Explanations are as in Fig. 1 except for: Ef *Ephydatia fluviatilis*.

Tlx genes encode related homeodomains and share the eh1 repression domain at the amino-terminal region (Fig. 2A). In bilaterians, genes of the Tlx family contribute to homeobox gene clusters, forming the vertebrate NKL and *Drosophila* 93DE complexes [28]. The phylogenetic analysis shows that the Tlx family is highly conserved in evolution and its members can be found from sponges to humans, but the positioning of *C. elegans* with a sponge and not within bilaterians indicates that this Tlx tree is not reflecting the evolutionary tree correctly. Recently, studies on the sponge *Ephydatia muelleri* show with a heterologous assay that the members of the Tlx family have structural and functional features conserved in phylogenetically distant groups [29]. Our discovery of *Tlx* in ctenophores confirms that this gene family could be one of the oldest of the extant animal-specific homeobox genes. Other fragments of ctenophore homeobox genes were isolated [30] but short PCR fragments are often not sufficient for analysis and could be contaminations from other species.

Multiple T-box and homeobox family members were apparently already present before the separation of the extant animal phyla. Similar conclusions were found with the *Wnt* gene family, which displays an unexpected ancestral diversity in sea anemones [31]; but also in this case the full richness of non-bilaterian diversity might only be found by comparative analysis of all four basal phyla.

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